This document provides a designated comparison method to standardize the measurement of ionized calcium made by ion-selective electrode (ISE) potentiometry. This system can be used to assign ionized calcium concentrations to a commercially available, serum-based material to improve the traceability and transferability of results for the measurement of ionized calcium in the clinical laboratory.

A standard for global application developed through the NCCLS consensus process.
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A Designated Comparison Method for the Measurement of Ionized Calcium in Serum; Approved Standard

Abstract

NCCLS document C39-A *A Designated Comparison Method for the Measurement of Ionized Calcium in Serum; Approved Standard* provides a candidate designated comparison method to standardize the measurement of ionized calcium made by ion-selective electrode (ISE) potentiometry. This system can be used to assign ionized calcium concentrations to a commercially available, serum-based material to improve the traceability and transferability of results for the measurement of ionized calcium in the clinical laboratory. This standard addresses the principle of the test, the assignment of ionized calcium concentrations to NIST Standard Reference Material 956a, the materials and methods used, and the results and conclusions of an interlaboratory study to assign the ionized calcium concentrations.


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A Designated Comparison Method for the Measurement of Ionized Calcium in Serum; Approved Standard

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Foreword

The measurement of ionized calcium in whole blood and serum is performed routinely as a service test in many clinical chemistry laboratories. A variety of manufacturers provides modern instrumentation, which allows the rapid measurement of ionized calcium in whole blood and serum with ion-selective membrane electrodes. These instruments, while highly sophisticated, differ in many ways from one manufacturer to another. All of these differences are known to affect the final ionized calcium result.

The purpose of developing this standard is to provide a candidate designated comparison method to standardize the measurement of ionized calcium made by ion-selective electrode (ISE) potentiometry and further, to use this system to assign ionized calcium concentrations to a commercially available, serum-based material to improve the traceability and transferability of results for the measurement of ionized calcium in the clinical laboratory. Development of this standard builds upon both clinical and industrial experience in laboratories around the world and is the result of many years of study of the analytical aspects of $i\text{Ca}^{2+}$ measurements.

The designated comparison method described in Appendix A of this document may be used to measure the concentration of ionized calcium in serum, not whole blood. The measurement of ionized calcium in whole blood by ISE potentiometry is known to be affected by the presence of erythrocytes. This effect is also present in commercial systems for the measurement of ionized calcium and is variable from one commercial system to another. This document does not address this problem. However, by standardizing the measurement of ionized calcium to a serum based reference material with concentrations assigned by the DCM, the interlaboratory variability for whole blood measurements of ionized calcium would be improved as well.

Key Words

Designated comparison method, ionized calcium, ion-selective electrode, potentiometry, SRM 956a
A Designated Comparison Method for the Measurement of Ionized Calcium in Serum; Approved Standard

1 Introduction

Ionized calcium (iCa$^{2+}$) has long been recognized as a better indicator of the physiological calcium status in human blood than total calcium.$^1$-11 The lack of a reference system for iCa$^{2+}$ has been recognized for several years.$^{12}$-15 As expected in the absence of a standardization procedure, reference intervals vary from location to location, even for the same type of commercial analyzer, because of differences among instruments, and from one type of commercial analyzer to another. The purpose of developing this standard is to provide a candidate designated comparison method (DCM) to standardize the measurement of ionized calcium made by ion-selective electrode (ISE) potentiometry; and further, to use this method to assign ionized calcium concentrations to a commercially available, serum-based material to improve the traceability and transferability of results for the measurement of ionized calcium in the clinical laboratory. Development of this method was based on both clinical and industrial experience in laboratories around the world, and is the result of many years of study of the analytical aspects of iCa$^{2+}$ measurements.$^6$-19

2 Scope

This document emphasizes the use of stable, deep-frozen (-50 °C), pooled serum (NIST SRM 956a) with iCa$^{2+}$ values assigned by a designated comparison method (DCM) as the key material which transfers accuracy for the measurement of ionized calcium. The substance concentration of iCa$^{2+}$ in this human serum-based material is determined on the basis of potentiometric comparison to defined standard solutions made from high-purity reference materials. These standards are aqueous solutions whose compositions are established by convention to contain known concentrations of ionized calcium at an ionic strength of 0.160 mol/kg. In general, preparation of these standards follows the recommendations of the Working Group on Selective Electrodes of the International Federation of Clinical Chemistry (IFCC).$^{15}$

The results of a multisite, interlaboratory study using NIST SRM 956a are reported. The objectives of this study were: 1) to show compatibility of the material with various commercial ionized calcium analyzers; and 2) to show usefulness of SRM 956a for providing uniformity to the measurement of ionized calcium in the clinical laboratory.

This document likewise provides specifications for the data acquisition hardware and software components of the ionized calcium DCM. Detailed information is included on the design of the potentiometric ISE and reference half-cells, liquid-liquid junction, and fabrication of tubular, calcium ion-selective membranes. Operating steps for system calibration, sample measurement, and data reduction are also described. Analytical specifications are described in terms of intralaboratory “within-run” and “day-to-day” imprecision to be expected when this technology is mastered.

3 Standard Precautions

Because it is often impossible to know what might be infectious, all human blood specimens are to be treated as infectious and handled according to “standard precautions.” Standard precautions are new guidelines that combine the major features of “universal precautions and body substance isolation” practices. Standard precautions cover the transmission of any pathogen and thus are more comprehensive than universal precautions which are intended to apply only to transmission of blood-borne pathogens. Standard precaution and universal precaution guidelines are available from the U.S. Centers for Disease Control and Prevention (Guideline for Isolation Precautions in Hospitals, Infection Control and Hospital Epidemiology, CDC, Vol 17;1:53-80.), [MMWR 1987;36(suppl 2S):2S-18S] and (MMWR 1988;37:377-
4 Principle of the Test

Ionized calcium in solution has been determined by several methods based on different analytical principles: 1) the biological frog heart method\(^1\); 2) spectrophotometry with calcium ion indicators\(^3,5,20,21\); and 3) potentiometry with calcium ion-selective electrodes, which responds to changes in thermodynamic activity of calcium ions.\(^3,22-26\) In theory the potentiometric cell responds to ion activity. However, in clinical chemistry, a convention has been adopted which allows standardization and reporting of results from ion-selective electrode potentiometry to be in units of concentration instead of activity. In subsequent sections of this document, concentration will be substituted for activity. See Section A2.1 in Appendix A for further information.

This proposed DCM is based on the third approach— the use of a potentiometric cell made up of two half-cells. The first half-cell is referred to as the “ISE half-cell” and consists of a calcium ion-selective membrane (M), an internal filling solution of fixed calcium ion activity, and a silver-silver chloride (Ag/AgCl) reference electrode (R1). The second half-cell is referred to as the “reference half-cell” and consists of a calomel internal reference electrode (R2) in a solution of saturated potassium chloride. The overall cell configuration may be written as:

\[
\text{R2 / KCl (sat’d) // test solution / M / internal filling solution / R1}
\]

\[
\text{reference half-cell \hspace{1cm} ISE half-cell}
\]

The connection between the two half-cells is made by a liquid-liquid junction between the test solution and saturated KCl, shown above as “//.”

On each side of the calcium ion-selective membrane (M), an electrical potential difference develops across the membrane-solution boundary. The calcium ion-selective membrane for this application is made in a tubular configuration.\(^17\) The outer side of the membrane is in contact with the internal filling solution of constant calcium ion activity and, hence, develops a constant potential. On the inner side of the membrane, the potential varies linearly with the logarithm of the calcium ion activity of the test solution with a response slope of 30.77 millivolts/decade at 37 °C, as governed by the Nernst Equation. Electrodes R1 and R2 are connected to an electrometer and PC-based data acquisition system.

The method is specifically designed for the anaerobic measurement of ionized calcium at 37 °C in serum pools stored frozen at –50 °C or below. In preparation for value assignment measurements, the serum is thawed anaerobically by a set protocol and transferred into the electrochemical cell. The ionized calcium concentration of the sample is calculated by measuring the potential generated by the cell in the presence of the unknown and comparing this value against the potentials generated by a set of known standards.

A measurement made with a potentiometric ion-selective electrode in an undiluted sample represents the concentration of the ion of interest in the water phase of the sample. This measurement bears a variable relationship to concentration of the ion in the entire sample volume as a function of the water content of the sample. Therefore, measurements made with the electrochemical cell described in the DCM should be considered concentrations of ionized calcium in the water phase of the sera. No attempt has been
made to correct for the nonwater fraction of the sample, which is composed primarily of protein and lipids.

Specifications of the proposed DCM and standard operating procedures for calibration and sample measurement are given in Appendix A.

5 Definitions

Calcium ion-selective membrane, n - The active element of the ISE half-cell that responds to changes in activity of calcium ion in solution. NOTE: A calcium ion-selective membrane typically contains an ionophore or ion-exchanger and a plasticizer in a plastic matrix such as polyvinyl chloride or polyurethane; other additives may be included to improve membrane functionality.

Data acquisition board, n - A device which collects and measures signals from sensors and sends them to a computer for processing.

Designated comparison method, DCM, n - A fully specified method(s), which, in the absence of a National Reference System for the Clinical Laboratory (NRSCL)-credentialled reference method, serves as the common basis for the comparison of “field” reference materials and methods, and for the development of principal assigned values (PAVs) or principal assigned characteristics (PACs) for an analyte or process.

Electrometer, n - A device which conditions (i.e., amplifies) a signal from a sensor and prepares it for collection and processing.

Faraday cage, n - An enclosure surrounding an electrochemical measurement system that serves as a shield against interference from ambient electromagnetic signals.

Ionized calcium (iCa$^{2+}$)/Free calcium, n - The concentration of calcium ions in whole blood that is not bound by protein or other molecules. NOTE: This parameter, also called “free” or “ionic” calcium, exists in the blood plasma water.

ISE half-cell, n - One-half of an overall electrochemical cell whose electrical potential varies in accordance with the Nernst Equation, as the concentration or activity of a single, desired species in solution is changed.

Liquid-liquid junction, n - The interface between two dissimilar solutions which typically serves as the point of connection between an indicator and reference half-cell.

Nernst equation, n - The expression which relates electromotive force (potential difference) of an electrochemical cell to the activity of chemical species in solution.

pH-adjusted ionized calcium, n - This calculated result is empirically based on a measured pH and ionized calcium concentration, where the ionized calcium is normalized to a pH of 7.40. NOTE: These calculations exclusively compensate for in vitro increases in pH due to loss of CO$_2$ and, therefore, help compensate for specimen handling errors; the pH-adjusted ionized calcium is automatically calculated by many of the commercial instruments currently available.

---

a Some of these definitions are found in NCCLS document NRSCL8—Terminology and Definitions for Use in NCCLS Documents. For complete definitions and detailed source information, please refer to the most current edition of that document.
Plasma, *n* - The liquid part of whole blood remaining after the separation of the cellular elements in a receptacle containing an anticoagulant, or separated by continuous filtration or centrifugation of anticoagulated blood in an apheresis procedure.

Potentiometry, *n* - A technique whereby the electromotive force (potential difference) of a cell can be related by means of the Nernst Equation to the activity of a single, desired species in solution.

Reference half-cell, *n* - One-half of an overall electrochemical cell whose potential is fixed and independent of the activity of any species in solution.

Response slope, *n* - The change in potential, usually given in millivolts, divided by the difference of the logarithms of two different ionized calcium concentrations. **NOTE:** The theoretical response slope of a calcium ISE toward calcium ion is 30.77 mV/log unit at 37 °C; the ideal millivolt change between 1.25 and 2.50 mmol/L ionized calcium in this designated comparison method is 9.255 mV.

Serum, *n* - The liquid remaining after treated whole blood has coagulated. **NOTE:** Observable after the clot and/or coagulum has retracted and/or has then been spun down in a centrifuge to separate the coagulum and cells from the liquid portion.

Total calcium, *n* - The entire calcium concentration in plasma, including ionized calcium and calcium bound to proteins or other molecules such as phosphate, bicarbonate, lactate, and citrate.

6 Assignment of Ionized Calcium Concentrations to NIST Standard Reference Material 956a

6.1 Description of the Material

Standard Reference Material® (SRM) 956a (National Institute of Standards and Technology (NIST), Gaithersburg, MD) is a certified reference material for the standardization of electrolytes in human serum. Presently, this material is certified by definitive methods for sodium, potassium, lithium, total calcium, and total magnesium. The specifications of this material, including the manufacturing procedures, are described in Appendix F. The material is frozen human serum stored at –50 °C or below. Based on knowledge of the stability of ionized calcium in human serum frozen at this temperature (see NCCLS document C31—Ionized Calcium Determinations: Precollection Variables, Specimen Choice, Collection, and Handling), the material could be useful for standardization of ionized calcium if a suitable method could be found for assignment of ionized calcium concentrations.

Approximately 750 ampules (250 ampules per each of three different concentration levels) from the September, 1995 production lot of SRM 956a were donated for this work by the manufacturer, Bayer, Inc. The Certificate of Analysis for this material issued by NIST is in Appendix B. Each ampule contains 2.0 ± 0.04 mL of human serum.

6.2 Description of the Approach

Ionized calcium concentrations have been assigned to NIST SRM 956a using the system described in Appendix A. This system is considered by the subcommittee to be a candidate designated comparison method (DCM), as described in NCCLS document NRSCL13—The Reference System for the Clinical Laboratory: Criteria for Development and Credentialing of Methods and Materials for Harmonization of Results. The present document describes most of the characteristics required for a DCM to be credentialled by the NRSCL Council. An important requirement, which has yet to be completed, is demonstration of the transferability of this system involving a minimum of three laboratories. Transferability studies are ongoing and are not described in this document.
The steps leading to the assignment of ionized calcium concentrations to SRM 956a are described below:

1) The performance of the DCM was evaluated over an extended period of time using stable, pooled serum.

2) Ionized calcium concentrations were assigned to SRM 956a using the DCM.

3) A multisite, interlaboratory study was conducted to determine if SRM 956a would be useful as an ionized calcium reference material to obtain uniformity among various commercial instruments in the field.

7 Materials and Methods

7.1 Preparation and Use of Stable Frozen Serum Pools for the Evaluation of the Candidate Designated Comparison Method (DCM)

The candidate DCM described in Appendix A was developed and evaluated in the clinical chemistry laboratory at Hartford Hospital, Hartford, CT by Drs. G.N. Bowers and R.B. McComb. Deeply frozen (-70 °C) pooled human serum, prepared and stored internally, provided the means to objectively determine short- and long-term analytical performance characteristics of the DCM. The source of the serum was discarded blood from patients undergoing therapeutic phlebotomy ordered by their physicians for polycythemia. The collection and processing procedures for preparation of the pool are described in NCCLS document C29—Standardization of Sodium and Potassium Ion-Selective Electrode Systems to the Flame Photometric Reference Method, Appendix A, and an earlier publication. In this document, this pool is designated as Human Serum Pool 97A.

Prior to measurements of ionized calcium, aliquots of serum pool 97A are thawed at 37 °C, transferred to an air-tight syringe and then adjusted to pH 7.400 ± 0.005 at 37 °C by equilibration with either small amounts of room air if the pH is below 7.395 or with 5% CO$_2$ if above 7.405. All pH measurements are made on a commercial pH/blood gas analyzer. This pH-adjusted serum in the syringe is held anaerobically at 0 to 4 °C, and a concurrent pH determination is made with each iCa$^{2+}$ measurement so that all ionized calcium results can be reported at pH = 7.400 as pH-adjusted ionized calcium.

The three test pools for the interlaboratory study, referred to in this document as materials D, E, and F, were from the same source and processed similarly to Pool 97A above. These pools were then transferred frozen to Bayer, Inc., Middletown, VA for ampuling under 5% CO$_2$ using the same procedure used for SRM 956a, described at the end of Appendix F.

7.2 Development of a Sample-Handling Protocol for SRM 956a

In preparation for value assignment measurements, ampules of the SRM 956a reference material must be taken from the frozen state at –50 °C to room temperature, while re-equilibrating the serum with the CO$_2$ concentration in the ampule headspace. A standard sample-handling protocol was needed to reach reproducible pH and ionized calcium concentrations between ampules and from location to location. A small group of subcommittee members conducted the experimentation necessary to understand the sample-handling requirements for the material in order to obtain reproducible pH and iCa$^{2+}$ values upon thawing. It was determined that 1 hour and 40 minutes was required for the contents of an ampule to thaw and reach room temperature after removal from the –50 °C freezer. Following this, the sample-handling variables which were examined are shown in the boxes below.
Thaw serum at ambient temperature for 1 hour and 40 minutes.

Shake ampule vigorously for ten seconds to re-equilibrate serum with CO\textsubscript{2} in the ampule headspace.

Open ampule.

Measure iCa\textsuperscript{2+}.

The results of these experiments were used to develop the sample-handling protocol for SRM 956a, which is part of Appendix C.

### 7.3 Measurement of pH in SRM 956a

Because of the influence of pH on ionized calcium, it was necessary to measure pH homogeneity among ampules of SRM 956a when the material is handled according to the protocol given in Appendix C. Ten ampules per level of SRM 956a were randomly selected and prepared for analysis according to the protocol. pH was measured using a system built and maintained at Chiron Diagnostics Corp., Medfield, MA and which conforms to the IFCC Reference Method for pH Measurement in Blood.\textsuperscript{28}

### 7.4 Assignment of Ionized Calcium Concentrations to SRM 956a

Assignment of ionized calcium concentrations to the three levels of SRM 956a using the candidate DCM and procedure described in Appendix A was carried out by G.N. Bowers, Jr. at Hartford Hospital, Hartford, CT. Data for each level of this material were obtained from runs carried out using at least six different calcium-selective membranes, shown to have acceptable iCa\textsuperscript{2+} recovery for serum pool 97A, and at least 20 ampules per level of material.

### 7.5 An Interlaboratory Study to Determine the Usefulness of SRM 956a as an Ionized Calcium Reference Material

An interlaboratory study (ILS) was conducted involving 19 industrial and clinical laboratories with an interest in ionized calcium standardization. The objectives of this multisite study were to determine if SRM 956a is compatible with the various types of commercial instrumentation available for measurement of ionized calcium, and if SRM 956a would be useful as a reference material to obtain uniformity among the various types of commercial instruments in the field. The list of participants in the ILS is shown in Appendix D. An effort was made to include at least one representative of all commercial instrumentation for the measurement of ionized calcium known to the subcommittee at the beginning of the study (April 1996). The ILS basically follows the recommendations given in ASTM Practice E 691-92.\textsuperscript{29} In addition to samples of SRM 956a, each laboratory was sent ampule samples of three other serum pools, prepared exclusively for the ILS (Materials D, E, and F). A standard protocol was supplied to all participating laboratories.
laboratories (Appendix C). The three additional pools were used as unknowns to test the standardization process, using the following procedure:

1. By use of concentrations of iCa\(^{2+}\) obtained from measurement of SRM 956a at each ILS site, a unique slope and intercept were calculated for each laboratory using a simple linear regression with the SRM 956a values from the DCM as the \(x\), or independent variable, and the values from each laboratory as the \(y\), or dependent variable.

2. This slope and intercept was then used to correct each laboratory’s results for three test pools.

3. Interlaboratory variability for the test pools was calculated before and after normalization of the data using analysis of variance (ANOVA).

8 Results

8.1 Measurement of Ionized Calcium in Serum Pool 97A

Table 1 shows the results of measurement of ionized calcium in Pool 97A carried out at Hartford Hospital over a period of 209 days using the candidate DCM described in Appendix A. Ten different calcium-selective membranes were used over this period of time. The grand mean is 1.301 mmol/L with a standard deviation of 0.006.

Table 1. Measurement of Ionized Calcium in Pool 97A Using the Candidate DCM

<table>
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<th>Date</th>
<th>Ca(^{2+}) membrane # (Days in Use)</th>
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<th>N (replicates /run)</th>
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<td>01/09</td>
<td>163 (5)</td>
<td>1.291</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>01/13</td>
<td>163 (9)</td>
<td>1.299</td>
<td>0.008</td>
<td>4</td>
</tr>
<tr>
<td>02/24</td>
<td>167 (2)</td>
<td>1.308</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>02/25</td>
<td>167 (3)</td>
<td>1.299</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>03/03</td>
<td>164 (2)</td>
<td>1.310</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>03/05</td>
<td>164 (4)</td>
<td>1.303</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>03/10</td>
<td>164 (9)</td>
<td>1.307</td>
<td>0.005</td>
<td>2</td>
</tr>
<tr>
<td>03/11</td>
<td>169 (2)</td>
<td>1.308</td>
<td>0.004</td>
<td>2</td>
</tr>
<tr>
<td>04/02</td>
<td>168 (2)</td>
<td>1.298</td>
<td>0.005</td>
<td>3</td>
</tr>
<tr>
<td>04/04</td>
<td>168 (4)</td>
<td>1.297</td>
<td>0.003</td>
<td>3</td>
</tr>
<tr>
<td>04/06</td>
<td>168 (6)</td>
<td>1.295</td>
<td>0.002</td>
<td>3</td>
</tr>
<tr>
<td>04/08</td>
<td>168 (8)</td>
<td>1.291</td>
<td>0.002</td>
<td>3</td>
</tr>
<tr>
<td>Grand Mean</td>
<td>1.301 0.006</td>
<td>63</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
8.2 Assignment of Ionized Calcium Concentrations to NIST SRM 956a

Table 2 shows the results of measurements of ionized calcium in the three levels of SRM 956a using the candidate DCM. At least 20 ampules per level of the SRM were used. The iCa\(^{2+}\) concentrations are *not* corrected to pH 7.40, but are measured and reported at the actual pH values shown in the table. Based on the known relationship between pH and iCa\(^{2+}\) for this material, it is estimated that the ampule-to-ampule variability in ionized calcium due to pH variability alone is less than 0.005 mmol/L.

**Table 2. Measurement of Ionized Calcium in NIST SRM 956a Using the Candidate DCM**

<table>
<thead>
<tr>
<th>SRM 956a Level</th>
<th>pH by IFCC Ref. Method(^{28})</th>
<th>Date</th>
<th>Ca(^{2+}) membrane #</th>
<th>Ca(^{2+}) by DCM mmol/L</th>
<th>SD</th>
<th>N (ampules/run)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (high Ca(^{2+}))</td>
<td></td>
<td>12/17/97</td>
<td>162</td>
<td>1.736</td>
<td>0.013</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>01/07/98</td>
<td>163</td>
<td>1.740</td>
<td>0.005</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>02/25/98</td>
<td>167</td>
<td>1.730</td>
<td>0.003</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>03/04/98</td>
<td>164</td>
<td>1.745</td>
<td>0.007</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>03/12/98</td>
<td>169</td>
<td>1.748</td>
<td>0.013</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>03/18/98</td>
<td>170</td>
<td>1.747</td>
<td>0.007</td>
<td>3</td>
</tr>
<tr>
<td><strong>Grand Mean ± SD</strong></td>
<td>7.496 ± 0.0065</td>
<td></td>
<td></td>
<td>1.741</td>
<td>0.007</td>
<td>21</td>
</tr>
<tr>
<td>II (mid Ca(^{2+}))</td>
<td></td>
<td>12/04/97</td>
<td>158</td>
<td>1.403</td>
<td>0.005</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>01/06/98</td>
<td>163</td>
<td>1.422</td>
<td>0.007</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>01/09/98</td>
<td>163</td>
<td>1.416</td>
<td>0.007</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>02/25/98</td>
<td>167</td>
<td>1.404</td>
<td>0.003</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>03/04/98</td>
<td>164</td>
<td>1.410</td>
<td>0.003</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>03/11/98</td>
<td>169</td>
<td>1.416</td>
<td>0.005</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>03/18/98</td>
<td>170</td>
<td>1.409</td>
<td>0.007</td>
<td>4</td>
</tr>
<tr>
<td><strong>Grand Mean ± SD</strong></td>
<td>7.532 ± 0.0050</td>
<td></td>
<td></td>
<td>1.411</td>
<td>0.007</td>
<td>25</td>
</tr>
<tr>
<td>III (low Ca(^{2+}))</td>
<td></td>
<td>12/16/97</td>
<td>162</td>
<td>1.087</td>
<td>0.004</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>01/08/98</td>
<td>163</td>
<td>1.098</td>
<td>0.004</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>02/24/98</td>
<td>167</td>
<td>1.088</td>
<td>0.005</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>03/03/98</td>
<td>164</td>
<td>1.093</td>
<td>0.005</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>03/11/98</td>
<td>169</td>
<td>1.087</td>
<td>0.007</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>03/18/98</td>
<td>170</td>
<td>1.093</td>
<td>0.007</td>
<td>3</td>
</tr>
<tr>
<td><strong>Grand Mean ± SD</strong></td>
<td>7.521 ± 0.0055</td>
<td></td>
<td></td>
<td>1.091</td>
<td>0.004</td>
<td>23</td>
</tr>
</tbody>
</table>

8.3 Results of the Interlaboratory Study

The raw data from the ILS are shown in Appendix E. Materials A, B, and C represent Levels I, II, and III of SRM 956a, respectively. Materials D, E, and F are three other serum pools, prepared for the ILS and used to test the standardization process. Following are the details of the materials sent out for the ILS.

- number of participating laboratories: 19
- total number of ampules sent to laboratories: 570 (30 per lab)
- ampules ruined (thawed) or lost in shipment: 90 (3 labs)
- ampules reported as broken on arrival: 15
• data points discarded as gross outliers (low pH, high iCa\(^2^+)\): 6 (see Section 9.3)
• samples lost due to instrument malfunction: 1
• total number of ampules shown in the data: 458 (16 labs)

Two of the laboratories chose to run the materials on more than one type of analyzer. These are represented in the data with a letter following the laboratory number and are treated in the data analysis as unique laboratories.

Analysis of variance was used to calculate the following components of standard deviation for Materials D, E, and F, before and after applying the normalization process described in Section 7.5: between-laboratories standard deviation (\(S_L\)), and the standard deviation for replicate measurements within laboratories (\(S_r\)). The results are shown in Table 3.

Table 3. Interlaboratory ANOVA Data (mmol/L) Before and After Normalization for Materials D, E, and F (Ionized Calcium Test Pools)

<table>
<thead>
<tr>
<th>Material</th>
<th>Material D</th>
<th>Material E</th>
<th>Material F</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S_L) (before correction)</td>
<td>0.078</td>
<td>0.100</td>
<td>0.097</td>
</tr>
<tr>
<td>(S_r) (before correction)</td>
<td>0.006</td>
<td>0.005</td>
<td>0.007</td>
</tr>
<tr>
<td>(S_L) (after correction)</td>
<td>0.036</td>
<td>0.051</td>
<td>0.032</td>
</tr>
<tr>
<td>(S_r) (after correction)</td>
<td>0.006</td>
<td>0.006</td>
<td>0.009</td>
</tr>
</tbody>
</table>

9 Conclusions and Recommendations

Table 3 shows, as expected, that the between-laboratories standard deviation (\(S_L\)) is much greater than the standard deviation for replicate measurements within laboratories (\(S_r\)). Normalizing the ionized calcium measurements in these test pools to SRM 956a lowers the between-laboratories standard deviation by a factor of two or greater.

Table 4 lists the recommended target values and uncertainties at the 95% level of confidence for ionized calcium in SRM 956a when material is handled according to the protocol of Appendix C.

Table 4. Assigned Ionized Calcium Concentrations for SRM 956a Using the Candidate DCM

<table>
<thead>
<tr>
<th>Level</th>
<th>Ionized Calcium mmol/L</th>
<th>Uncertainty* (\pm) mmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Level I</td>
<td>1.741</td>
<td>0.018</td>
</tr>
<tr>
<td>Level II</td>
<td>1.411</td>
<td>0.017</td>
</tr>
<tr>
<td>Level III</td>
<td>1.091</td>
<td>0.010</td>
</tr>
</tbody>
</table>

*95% Confidence Interval
9.1 Procedure for Standardization of Commercial Instruments for the Measurement of Ionized Calcium to SRM 956a

9.1.1 System Calibration and Sample Analysis

This procedure is essentially the same as that detailed in NCCLS document C29—Standardization of Sodium and Potassium Ion-Selective Electrode Systems to the Flame Photometric Reference Method. Refer to Section 6.2 and Appendixes B and C of NCCLS document C29 for further detailed information.

The protocol below should be used to standardize a commercial analyzer for the determination of iCa$^{2+}$ to SRM 956a. The analyzer should be calibrated and operated according to the manufacturer’s instructions.

1. SRM 956a should be prepared for analysis according to Section C3 of Appendix C of this document.

2. Since replicate determinations of each level of material will be necessary, to conserve material, the contents of each ampule should be transferred to a syringe before analysis while minimizing contact with air.

3. During analysis, bubbles formed in the syringe should be removed, and the syringe should be kept capped between analyses.

4. Each of the three levels of the SRM should be run for a total of 15 replicates per level on the analyzer.

5. Replicate measurements should be in random order with respect to concentration.

For this protocol to be valid, the coefficient of variation (%CV, within-run) should be less than or equal to 1.5% at all levels. The means of the determinations from the iCa$^{2+}$ analyzer and the assigned iCa$^{2+}$ concentrations for SRM 956a (Table 4) should agree to within ±1% at all concentrations. See Section 9.1.2 if the ±1% agreement cannot be obtained. This will guarantee, at the 95% level of confidence, that the true difference between the analyzer and the SRM 956a assigned values is within ±2%. See Appendix B in NCCLS document C29—Standardization of Sodium and Potassium Ion-Selective Electrode Systems to the Flame Photometric Reference Method, for further details.

9.1.2 Slope and Intercept Correction Factors

1. If ±1% agreement is not obtained above, then the two sets of data should be analyzed by linear regression, with the iCa$^{2+}$ assigned concentrations for SRM 956a (Table 4) as the x, or independent variable and the means of the determinations from the iCa$^{2+}$ analyzer as the y, or dependent variable.

2. The best fit line should be adjusted to a slope of 1 and an intercept of 0, using the appropriate correction factors. An example of this process is given in NCCLS document C29—Standardization of Sodium and Potassium Ion-Selective Electrode Systems to the Flame Photometric Reference Method, Appendix C.

3. All subsequent data should be corrected using the slope and intercept correction factors. Certain commercial analyzers allow the slope and intercept correction factors to be entered into the instrument’s microprocessor at the user level. If this feature is not available, the corrections can be done manually or through an appropriate data management system.

9.2 Stability of Ionized Calcium in SRM 956a

Data from one study has suggested that there is little change in the concentration of ionized calcium in serum during long-term storage at temperatures of −50 °C or below (Bowers, GN Jr., Hartford, Conn.,
personal communication [1996]). The values reported for SRM 956a from the candidate DCM, shown in Table 2, were obtained over a three-month period (December 1997 to March 1998). These ionized calcium data are in agreement with preliminary data taken at the beginning of this study (September 1996 data not shown). The intent is to continue to monitor the pH stability of this material at six-month intervals. Information regarding the assigned values for ionized calcium in SRM 956a and any additional information regarding stability of ionized calcium in this material will be communicated as an addendum to the NIST Certificate of Analysis for SRM 956a.

9.3 Precaution

A small number of ampules were reported to have arrived broken during the interlaboratory test (see Section 8.3). These were discarded from the data set. Another small number of ampules were discarded due to gross outliers (low pH, high iCa\(^{2+}\)). These outliers are believed to have been caused by small cracks in the ampules which allowed contamination of the serum by CO\(_2\) from dry ice during shipment. As an additional safeguard from contamination by CO\(_2\), the product, as shipped by NIST, will have two additional barriers to CO\(_2\) when it is packaged with dry ice. A shipping test of SRM 956a from NIST in this package configuration to eight laboratories in the United States and Europe was conducted in September 1996 to address this problem. No broken ampules or outliers were reported during the shipping test. At this time the breaks and outliers observed during the ILS are believed to have been caused by poor packaging or mishandling of those ampules during that test only. However, to guard against the possibility of obtaining a defective ampule, any ampule whose contents are 0.1 pH unit or more lower than the pH values from Table 2 for each level of the SRM should be omitted from the data. Any ampules which are broken or have visible cracks should, of course, not be used.
References


Appendix A. Proposed Designated Comparison Method for Ionized Calcium in Serum: Assembly and Operation

NOTE: The specific product manufacturer details in this appendix are provided for informational purposes only and do not imply an endorsement by NCCLS.

A1 Apparatus

The ionized calcium DCM measurement apparatus is custom designed, but can be assembled using components obtained from the usual commercial sources of laboratory and electronic supplies. See Figure A for an overall schematic diagram of the system. One exception is the in-house preparation of the tubular calcium-selective membrane and the associated glass tubing which serves as both membrane support and sample line. The procedure for preparing the tubular membrane is given in detail in Section A2.6 and can be readily mastered. The bending of narrow-bore glass tubing and fabrication of the plastic tops for each half-cell require no exceptional skills. The electrometer and data acquisition system can easily be assembled with a basic knowledge of electrical engineering, but care must be taken to ensure the data acquisition printed circuit board (Section A1.3.2) meets specifications and is compatible with the data acquisition software used in the computer.

A1.1 ISE Half-Cell

1. A water-jacketed, 250-mL glass cell (Ace Glass, Vineland, NJ), with an inside diameter of 65 mm and an inside height of 89 mm, is connected to a circulating water bath to maintain the filling solution (FS, Section A2.4.1 below) in the cell at 37.00 ± 0.05 °C. A clear plastic cover (10 cm long x 9 cm wide x 1 cm high) rests on top of the cell.

2. A clear plastic cylinder (4 cm diameter x 3 cm high) is bonded to the top of the cover at center. The cylinder has three vertically bored holes, two (3 mm diameter) for the glass capillary tubes which position the ISE membrane in the FS and serve as the sample flow path, and one (14 mm diameter) that positions the Ag/AgCl electrode behind the ISE membrane within the FS. The two small holes for the glass capillary tubes are separated by 20 mm (center-to-center) on an axis that crosses the main front/back axis at a right angle 13 mm from the front of the plastic support cylinder. The 14-mm hole is centered on the front/back axis, 10 mm from the back.

3. The internal Ag/AgCl electrode is a double-junction Ag/AgCl electrode (Fisher Scientific, Pittsburgh, PA) and the lead from this electrode is labeled “S” (signal) and attached to the signal input terminal of the electrometer circuit.

4. The glass capillary tubes (2.8 to 3.0 mm outer diameter x ~1.6 mm inner diameter) should be cut, bent, and positioned in the cell as shown in Figure A.

5. The outer diameter of the two pieces of tubing to receive the membrane should be tapered slightly at the ends to an outer diameter of ~2.2 mm.

6. The glass capillary tube on the right (labeled “sample in”) extends 25 mm above the plastic cylinder for attachment of a Tygon input line. The same piece of tubing extends 60 mm below the plastic cover, makes a right angle bend, and extends 20 mm further to a second right angle bend that sends the tapered end vertically up for 10 mm, directly under another small hole in the plastic cover.

7. The tapered end of a second glass capillary tube is positioned directly above the first and a 10-mm gap is left for attachment of the ISE membrane.
(8) The glass tubing on the left extends vertically up through the filling solution, the plastic cylinder, and extends another 25 mm above the top of the plastic cylinder.

(9) The plastic sample bridge line is attached to this glass tubing on the left side of the ISE half-cell.

(10) Two small rubber o-rings are used to secure the glass tubing to the plastic cover at the top of the ISE half-cell.

(11) The two 20-cm plastic input and bridge lines are surgical Tygon type PVC tubing which measure 0.8 mm inside diameter and 2.4 mm outside diameter. These lines are held tightly to the glass capillary tubing with 3-cm-long silicone rubber tubing (1.6 mm inside diameter and 4.8 mm outside diameter).

(12) At the distal end of the 20-cm input line on the right side of the ISE half-cell, an 8- to 10-cm length of hypodermic stainless steel tubing is inserted within the Tygon tubing in order to aspirate samples directly from a syringe, test tube, or glass ampule (not shown in Figure A). A small hemostat is used to close this line by clamping just above the end of the inserted steel tube.

(13) To prevent damaging the Tygon tubing, a 2-cm piece of silicone rubber tubing is slipped over the Tygon to the area where the clamp is applied.

**A1.2 Reference Half-Cell**

(1) A water-jacketed, 400-mL glass cell (Ace Glass, Vineland, NJ), with an inside height of 112 mm and an inside diameter of 75 mm, is likewise connected to the circulating water bath, in series with the ISE half-cell, to maintain temperature control to 37.00 ± 0.05 °C.

(2) The cell is filled with a solution of saturated potassium chloride to a level about 1 cm below the level of the filling solution in the ISE half-cell.

(3) The cell is placed on an air-driven turbine magnetic stirrer (Fisher Scientific, Pittsburgh, PA) which rotates a small Teflon-coated magnetic stir bar within the cell at about 150 rpm (not shown in Figure A). Excess solid potassium chloride is always present in the reference half-cell so that with constant stirring, the solution remains saturated at 37 °C.

(4) A clear plastic cover (12 cm x 12 cm x 5 cm high) with a 45-mm diameter hole at center rests on top of the cell. This hole holds a rubber stopper (top 50 mm in diameter, bottom 40 mm in diameter, thickness 25 mm). Vertical holes bored into the rubber stopper position a standard calomel reference electrode (Fisher Scientific, Pittsburgh, PA) in the rear and the glass capillary bridge tube in front.

(5) The glass capillary tube attached to the left end of the plastic sample bridge makes a right-angle bend at 4 cm from the end of the plastic line and goes down through the rubber stopper into the KCl, and has a position so that the liquid junction at the end of the glass tube points upward and is 1 cm below the surface of the KCl and at least 1 cm away from the tip of the reference electrode.
Figure A. Ionized Calcium Designated Comparison Method Instrumentation (not drawn to scale)
(6) When calibrator or sample is to be introduced into the cell, the end of the glass bridge tube is temporarily removed from the KCl and attached to an external aspiration line fitted with a silicone rubber tube at the end.

(7) A gentle suction which introduces calibrator or sample into the cell at a rate of 4 to 5 mL/min is created by a small peristaltic pump.

(8) The wire lead from the electrode in the reference half-cell is labeled “R” (reference) and is attached to the reference input terminal of the electrometer circuit.

A1.3 Signal Acquisition Subsystems

A1.3.1 Electrometer

In order to prevent attenuation of the signals from the ISE and reference half-cells, a high-input impedance electrometer is necessary to buffer the signal before it is acquired by the data acquisition board that is installed in the computer. The electrometer specifications required to accurately acquire the electrical signal from the iCa\(^{2+}\) cell are:

- input bias current: 2.5 x 10\(^{-13}\) Ampere maximum
- input resistance: 10\(^{12}\) Ω minimum
- input offset voltage vs. temperature: 5μV/°C maximum
- input capacitance: 1 nF maximum
- input type: differential input, floating with respect to ground
- resistance to ground: 10\(^{12}\) Ω minimum
- commonmode rejection ratio: 80 dB

Electrometers meeting these specifications are commercially available, for example, from World Precision Instruments, Sarasota, FL (Model VF4, four-channel buffer amplifier). However, the device can be easily assembled by one with a fundamental knowledge of electrical engineering.

A1.3.2 Data Acquisition Board

Following conditioning of the signals by the electrometer (see Section A1.3.1), the data is acquired using a plug-in data acquisition board that is installed in a personal computer. Three specifications for the data acquisition boards are important: 1) the number of bits into which the data acquisition board can resolve the input signal; 2) the resolution specified in volts; and 3) the electronic nonlinearity specification. For an ionized calcium DCM, a 16-bit data acquisition board is necessary in order to obtain the necessary resolution. Data acquisition boards typically have adjustable gains which allow the input range of the board to be closely matched to the output range of the electrometer. Linearity error may be specified either as a percentage of the full scale resolution (FSR) or as the number of significant bits. The data acquisition board specifications are as follows:

- number of bits: 16
- resolution: 10μV minimum
- nonlinearity: ±0.02% FSR maximum or ±1 least significant bit (LSB)

Data acquisition boards meeting the above specifications are available from several commercial suppliers of electronic test and measurement equipment.*

A1.3.3 Computer and Data Acquisition Software

An IBM-compatible (486DX or equivalent) personal computer may be used to acquire data. There are several commercially available software packages for data acquisition which may be used. Many contain features which allow the acquired signal to be displayed graphically in real time. An important feature of the data acquisition software for the ionized calcium DCM is the rate at which data are collected. It is recommended that data be collected at a sampling rate not less than five points/second. The data may then be processed and reported as a one-second average of the multiple points/second. The standard deviation of each second of data is useful to assess the noise of the measurement.

A1.3.4 Grounding and Shielding

For low-level measurement applications, an effective grounding and shielding system is crucial. A Faraday cage that is connected to earth ground shields the electrochemical cell. The leads from the electrodes to the electrometer are twisted-shielded cable, and their length is kept as short as possible. The shielding on the cable is connected to the Faraday cage. Twisted-shielded cable is effective at limiting noise pick-up on signal conductors, particularly from lower-frequency noise sources. The cable has polyethylene insulation which has excellent volume resistivity, flexibility, and resistance to water absorption. Teflon™ is a superior insulating material and may also be used. The electrometer is housed in an aluminum enclosure which is connected to the input cable shield.

An external ±12-Volt DC power supply with additional filtering is used to power the electrometer. The power supply is packaged in an aluminum enclosure that is connected to the ground lead of the AC input. The output common is not at the same potential as the case ground and is earthed separately.

The high-speed digital electronics inside personal computers create high noise levels. The grounding and shielding system for the electrochemical cell and electrometer should never be grounded to the computer case.

A2 Materials and Reagents

A2.1 Matrix of Ionized Calcium Standards

The ionized calcium DCM is standardized using a set of aqueous solutions whose compositions are established by convention to contain known concentrations of ionized calcium at an ionic strength of 0.160 mol/kg. This convention includes the assumption that the average ionic strength of blood-plasma water is 0.160 mol/kg.† Because ionic strength is the primary variable affecting the activity coefficient of ionic species in solution, controlling the ionic strength of the calibrating solutions to 0.160 mol/kg should allow the activity coefficients of ionic species in the calibrators to approximate those in blood-plasma water. Both calibrations and the expression of measured results in plasma or serum may then be made in units of concentration instead of activity. In general, this convention and preparation of these standards follow the recommendations of the IFCC Working Group on Selective Electrodes for preparation of primary standardization solutions,‡ herein called “primary calibration solutions.” These solutions contain only primary standard for the calcium ion and ultrapure material for the only other solute, sodium chloride (see Section A2.1.1), and are prepared by gravimetric techniques. Secondary calibration solutions are also used. These secondary calibration solutions make use of the same source for calcium.


ion and sodium chloride, but also contain potassium and magnesium chloride, in addition to a low concentration of HEPES buffer and a nonionic surfactant, Triton X-100®.

**Table A1. Composition of Ionized Calcium Standards**

<table>
<thead>
<tr>
<th></th>
<th>Primary Calibration Solutions (I = 160 mmol/kg)</th>
<th>Secondary Calibration Solutions (I= 160 mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$c\text{Ca}^{2+}$</td>
<td>$m\text{Ca}^{2+}$</td>
</tr>
<tr>
<td>1.25</td>
<td>1.256</td>
<td>156.25</td>
</tr>
<tr>
<td>2.50</td>
<td>2.526</td>
<td>152.50</td>
</tr>
<tr>
<td>$c = \text{molar concentration (in mmol/L)}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$m = \text{molal concentration (in mmol/kg)}$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A2.1.1 Reagent Sources

The following materials were used in preparation of standards: calcium carbonate (SRM 915) and HEPES (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]) (SRM 2181) from the National Institute of Standards and Technology (NIST), Gaithersburg, MD; sodium chloride (#3642.5, J.T. Baker, Phillipsburg, PA); potassium chloride (#6858, Mallinckrodt, Paris, KY); magnesium chloride hexahydrate (#25,577.7, Aldrich Chemical Co., Milwaukee, WI); Triton-X-100 (#BP151-100, Fisher Scientific, Pittsburgh, PA); hydrochloric acid (360 g/kg, #HX0601-13) and sodium hydroxide (500 g/kg, carbonate free, #SX0597-3, EM Science, Gibbstown, NJ).

A2.2 Primary Calibration Solutions

A2.2.1 Preparation of 100.0 mmol/L (23 °C) Calcium Chloride (Stock Calcium Solution)

This stock solution was prepared by the procedure of Boink et al.‡ at a temperature of 23 ± 2 °C and a relative humidity of 50 ± 5%.

1. All glass apparatus must be cleaned in hydrochloric acid (1:2 dilution of concentrated hydrochloric acid, or approximately 6 mol/L), rinsed in deionized water, and dried before use.

2. Calcium carbonate (0.1000 moles [10.009 g], dried 1 hour at 105 °C) and 100 mL of deionized water are transferred to a conical flask and 0.22 moles (44.56 g of the 1:2 dilution) of hydrochloric acid is slowly added.

3. When the calcium carbonate has dissolved, the solution is boiled for 15 minutes.

4. The mixture is transferred quantitatively to a calibrated 1,000 mL (23 °C) volumetric flask and filled to the total volume with CO₂ free deionized water.

5. The hydrochloric acid content of this solution is determined by titration to phenolphthalein end-point.
The density of this solution has been found to be 1.0068 g/cm³ (weighed in ambient air) at 23 °C. As the density is known, the stock calcium solution can also be prepared by weight alone. An analytical balance with a resolution of 0.0001 g was used. The same balance was used in preparing the calibrators below.

A2.2.2 Preparation of 2.500 mmol/L Ca²⁺ (High Aqueous Calibrator: I = 160 mmol/kg)

1. To a tared, 1-L Class A volumetric flask was added 25.170 g of 100.0 mmol/L stock calcium solution, 500 mL of CO₂ free deionized water, and 152.5 mmoles (8.9121 g) of NaCl.

2. The mass of NaCl actually added to the solution must be reduced by the small amount of NaCl formed when the HCl associated with the CaCl₂ stock solution is neutralized with NaOH below. This amounts to approximately 0.5 mmoles, the exact amount being determined previously by titration in A2.2.1. For example, if titration reveals that there is 0.02 mol/L of free HCl in the CaCl₂ stock calcium solution, then 0.5 mmoles (25 x 0.02) of HCl are present in the calibrator at this point. Therefore, the mass of NaCl required would be (152.5-0.5) x 58.44 or 8.8829 g. The 0.5 mmoles of Na⁺ is added as 1.0 mol/L NaOH.

3. The solution is swirled to mix, and the total weight is adjusted to 1006.2 g with deionized water.

The pH of this calibrator should be in the range of 5.5 to 7.0 in order to minimize pick up of CO₂ from the atmosphere or cause CO₂ release from the serum sample during analysis.

A2.2.3 Preparation of 1.250 mmol/L Ca²⁺ (Low Aqueous Calibrator: I = 160 mmol/kg)

1. To a tared, 1-L Class A volumetric flask was added 12.585 g of 100.0 mmol/L stock calcium solution, 500 mL of CO₂ free deionized water, and 156.25 mmoles of NaCl.

2. The mass of NaCl actually added to the solution must be reduced by the small amount of NaCl formed when the HCl associated with the CaCl₂ stock solution is neutralized with NaOH below. This amounts to approximately 0.25 mmoles, the exact amount being determined previously by titration in Section A2.2.1.

3. The 0.25 m mole of Na⁺ is added as 1.0 mol/L NaOH.

4. The solution is swirled to mix, and the total weight is adjusted to 1006.2 g with deionized water. The pH of this calibrator should be in the range of 5.5 to 7.0 in order to minimize pickup of CO₂ from the atmosphere or cause CO₂ release from the serum sample during analysis.

A2.3 Secondary Calibration Solutions (Working Calibrators)

A2.3.1 Calibration of Glassware

The preparation of secondary calibration solutions uses volumetric techniques. It is first necessary to recalibrate a 1-L volumetric flask so that volumes measured at 23 °C will occupy exactly 1 L at 37 °C.

The following procedure is used to set the 37 °C, 1-L mark:

1. To a 1-L, tared volumetric flask, add exactly 992.4 g water (weight of 1 L at 37 °C weighed in air).

2. Bring the temperature of this water to 23 °C and inscribe a line at the meniscus of the water using a glass-marking pencil.
(3) Check the accuracy of this calibration by immersing the flask in a 37 °C bath and confirming that the meniscus is now at the original calibration line.

A2.3.2 Preparation of 2.500 mmol/L Ca\(^{2+}\) (High Secondary Calibrator: I = 160 mmol/L; **NOTE:** Ionic strength in these secondary calibrators is expressed in mmol/L following the IFCC recommendation.\(^6\))

(1) To a 1-L volumetric flask that has been recalibrated to hold exactly 1,000 mL at 37 °C, was added 25.00 mL of 100.0-mmol/L stock calcium solution, 100 mg (0.5 mmoles) MgCl\(_2\)•6H\(_2\)O, 335 mg (4.5 mmoles) KCl, 238 mg (1.0 mmoles) HEPES, 500 mL of CO\(_2\) free deionized water, and 146.5 mmoles NaCl.

(2) The mass of NaCl actually added must be reduced by the small amounts of NaCl and NaHEPES formed when the acid associated with the CaCl\(_2\), and the HEPES buffer, is neutralized with NaOH below. This amounts to approximately 1.0 mmole for the combined neutralization of free HCl and HEPES, the exact amount being determined by titration.

(3) The appropriate amount of NaOH needed to neutralize the free acid is added next, followed by 0.3 g (10 drops) of Triton X-100\(^\circledR\).

(4) The total volume is adjusted to the 37 °C, 1-L mark with deionized water.

(5) The solution is mixed to dissolve the surfactant.

The pH of this calibrator should be in the range of 7.3 to 7.4.

A2.3.3 Preparation of 0.000 mmol/L Ca\(^{2+}\) (Blank for Secondary Calibrators: I = 160 mmol/L; **NOTE:** Ionic strength in these secondary calibrators is expressed in mmol/L following the IFCC recommendation.\(^6\))

(1) To a 1-L volumetric flask that has been recalibrated to hold exactly 1,000 mL at 37 °C, was added 100 mg (0.5 mmoles) MgCl\(_2\)•6H\(_2\)O, 335 mg (4.5 mmoles) KCl, 238 mg (1.0 mmoles) HEPES, 500 mL of CO\(_2\) free deionized water at 23 °C, and 154.0 mmoles of NaCl.

(2) The mass of NaCl actually added at this point is reduced by the small amount of NaHEPES formed when the uncharged buffer reacts with NaOH in the next step. This amounts to approximately 0.55 mmoles.

(3) The NaOH is added, followed by 0.3 g (10 drops) of Triton X-100\(^\circledR\).

(4) The total volume is adjusted to the 37 °C, 1-L mark with deionized water at 23 °C.

(5) The solution is mixed to dissolve the surfactant.

The pH of this calibrator should be in the range of 7.3 to 7.4.

A2.3.4 Preparation of 1.250 mmol/L Ca\(^{2+}\) (Low Secondary Calibrator: I = 160 mmol/L; **NOTE:** Ionic strength in these secondary calibrators is expressed in mmol/L following the IFCC recommendation.\(^6\))

(1) A 500-mL, Class A volumetric flask is filled exactly to the TC mark with the 2.500 mmol/L Ca\(^{2+}\) High Secondary Calibrator at 23 ± 2 °C.

(2) The contents of the flask are quantitatively delivered into a 1-L, Class A volumetric flask with three wash-outs with the 0.000 mmol/L Ca\(^{2+}\) blank.

(3) The volume is then adjusted to exactly 1 L with additional 0.000 mmol/L Ca\(^{2+}\) blank.

(4) The solution is capped, mixed well, and stored at room temperature.

A2.3.5 Preparation of Other Secondary Calibrator Solutions

A series of near-bracketing secondary calibrators is easily prepared using the 2.500-, 1.250-, and 0.000- mmol/L Ca\(^{2+}\) secondary calibrators prepared above. For example, 2.250-, 2.000-, 1.750-, and 1.500- mmol/L Ca\(^{2+}\) secondary calibrators can be prepared by volumetrically adding 10.00, 20.00, 30.00, or 40.00 mL of 0.000-mmol/L, respectively, into 100-mL volumetric flasks and bringing each to the mark with the 2.500-mmol/L secondary calibrator. Similar use of the 1.250-mmol/L secondary calibrator would yield 1.125-, 1.000-, 0.875-, and 0.750-mmol/L Ca\(^{2+}\) secondary calibrators.

A2.4 Other Solutions

A2.4.1 ISE Half-Cell Filling Solution (1.250 mmol/L CaCl\(_2\), I=160 mmol/L)

(1) To a 1-L volumetric flask that has been recalibrated to hold exactly 1,000.0 mL at 37 °C, was added 12.50 mL of 100.0-mmol/L CaCl\(_2\) stock, 100 mg (0.5 mmoles) MgCl\(_2\cdot6\)H\(_2\)O, 335 mg (4.5 mmoles) KCl, 238 mg (1.0 mmoles) HEPES, 500 mL of CO\(_2\) free deionized water, and 150.25 mmoles of NaCl.

(2) The mass of NaCl actually added must be reduced by the small amounts of NaCl and NaHEPES formed when the acidity associated with the CaCl\(_2\) and HEPES is neutralized with NaOH below. This amounts to approximately 1.0 m mole for the combined neutralization of free HCl and HEPES, the exact amount being determined by titration.

(3) The NaOH is added next; the total volume is adjusted to the 37 °C, 1-L mark with deionized water.

The pH of this solution should be in the range of 7.3 to 7.4. The solution is identical to that prepared in Section A2.3.4, except it contains no Triton X-100\(^\circledR\).

A2.4.2 Saturated Potassium Chloride

A solution of potassium chloride in water, saturated at 37 °C, is used to fill the reference half-cell. This solution can be obtained from commercial suppliers of ISE equipment or may be prepared directly in the reference half-cell by adding solid KCl to distilled-deionized water until solid KCl remains after continuous stirring at 37.0 °C. The molality of this saturated solution is 5.26 mol/kg with respect to KCl at 37.0 °C. It is recommended that this solution be changed weekly during periods of system use.

A2.4.3 Cleaning Solution for Sample Line

It is recommended that a cleaning solution be run through the sample line daily, if the system has been exposed to serum. This procedure prevents build-up of protein on the membrane and in the sample line. The solution should consist of 22.8 kU/mL pepsin (Anson units) in a background of 140 mmol/L NaCl, 4.0 mmol/L KCl, 1.25 mmol/L CaCl\(_2\), 0.075 molar hydrochloric acid, and 500 ppm Triton X-100\(^\circledR\). The solution is introduced into the sample line at the end of serum analyses for the day, and allowed to remain
in contact with the sample line and membrane for approximately five minutes. Following this, the solution is flushed from the line using copious amounts of the 1.25 mmol/L ionized calcium secondary calibrator.

A2.5 Summary of Selectivity Studies of the Calcium Ion-Selective Membrane

The formulation of the calcium ion-selective membrane that forms the basis of the system described in this Appendix has not changed since the early 1980s. The selective recognition of the calcium ion is based on the action of the ionophore (-)-(R,R)-N,N′-bis[(11-ethoxycarbonyl)undecyl]-N,N′-4,5-tetramethyl-3,6-dioxaoctane-diamide (ETH 1001) which is in a membrane matrix of plasticized poly(vinyl) chloride. When establishing accuracy of a measurement with any ion-selective membrane electrode, selectivity over other ions of like charge in the sample is the key attribute. Available selectivity data for the calcium ion-selective membrane based on ETH 1001 are summarized below.

Selectivity for the Calcium Ion-Selective Membrane Based on the Ionophore ETH 1001 Over Other Serum Cations Expressed as Log $K_{\text{ca}/j}$

("Required" selectivity is for a maximum interference of 1% by interfering cation $j$, at a concentration representing the middle of the normal range in serum for the primary ($\text{Ca}^{2+}$) and interfering ion.)

<table>
<thead>
<tr>
<th>Interfering Cation j</th>
<th>$\text{Na}^+$</th>
<th>$\text{K}^+$</th>
<th>$\text{Mg}^{2+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Required</td>
<td>-3.6</td>
<td>-0.6</td>
<td>-1.9</td>
</tr>
<tr>
<td>Measured†</td>
<td>-3.4</td>
<td>-3.5</td>
<td>-4.1</td>
</tr>
<tr>
<td>Measured (this work)</td>
<td>-3.7</td>
<td>-3.5</td>
<td>-3.6</td>
</tr>
</tbody>
</table>

The more negative the log $K$ value, the less the interference from a given cation. Therefore, sodium in the sample, at a concentration with the normal range, will produce approximately 1% interference in the measured ionized calcium if left uncompensated. However, with sodium in the calibration solutions at a concentration approximately equivalent to the samples, the interference is present during calibration and sample measurement, and the majority of the error will cancel. The resulting net error is negligible as long as the sodium concentration of the sample is within the physiological range.

A2.6 Preparation of the Calcium Ion-Selective Membrane

A2.6.1 Membrane Materials (see Note)

NOTE: a) Calcium-selective membranes using ionophores other than ETH 1001, including charged calcium ion exchangers, have been reported in the literature and are used in limited commercial applications. However, ETH 1001 is in widespread commercial use and has a history of nearly six years of use in the system proposed here. ETH 1001 has recently become available from another commercial source, Selective Technologies Inc., Flint, MI and is sold under the trade name CA 1001; b) Although the majority of commercially available analyzers for the measurement of ionized calcium in blood use a calcium ISE based on polyvinyl chloride, we have demonstrated that substitution of the type of polyurethane listed here for polyvinyl chloride is preferred because of its superior compatibility with

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components in the serum matrix and because it has no influence on other ISE properties such as slope, response time, and selectivity.\(^a\)

The following materials were used to construct the calcium ion-selective membrane: nitrophenyl octyl ether (plasticizer, Cat. No. 73732); calcium ionophore I (ETH 1001, Cat No. 21192 at least 95% by HPLC); and potassium tetra(4-chlorophenyl)borate (KClPB, Cat. No. 60591), all from Fluka Chemical Co., Ronkonkoma, NY. Polyurethane (linear segmented, aliphatic polyether-based, Tecoflex SG80-A) was from Thermedics, Woburn, MA. Tetrahydrofuran (THF, 99.9%, Cat. No. 18,652-2) was purchased from Aldrich Chemical Co., Milwaukee, WI.

\[
\text{ETH 1001 Calcium Ionophore}
\]

A2.6.2 Membrane Starting Solution

(1) A 15-mL, 28 x 58 mm screw-cap vial (#87793-20 from Scientific Products, McGraw Park, IL) was tared on a microbalance.

(2) A Teflon liner (#2390505, Thomas Scientific, Swedesboro, NJ) was inserted in the cap to prevent attack of the neoprene by the THF solvent.

(3) After temperature equilibration was established, 15 ± 2 mg of calcium ionophore, ETH 1001, was added to the vial from a 10-mL glass syringe (Microliter #701, Hamilton Co., Reno, NV).

(4) The vial was removed from the balance for this addition, the ionophore was added in two to three applications from the syringe, and the amount present determined after each addition.

After the exact weight of the ionophore was determined, the amounts of KClPB, plasticizer, and polyurethane to be used were calculated as follows:

1. The final concentration of ionophore is 2.0% w/w of the final membrane.
2. The ratio of weight of polyurethane (PU) to plasticizer (PL) is 2 to 3.
3. The molar ratio of KClPB to ionophore is 0.50.
4. The ratio of the final volume of THF to the combined weight of nonvolatile membrane components is 0.00542 (mL/mg).

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These conditions are satisfied by the following equations:

\[ 1. \] Final membrane weight = weight of ionophore/0.020.
\[ 2. \] PL + PU + (weight ionophore + weight KCIPB) = final membrane weight, but PL = 3/2(PU) and molar ratio of KCIPB to ionophore = 0.50.

An example of these calculations is given below for 10 mg of ionophore:

\[ \begin{align*}
PL + PU + (weight \text{ ionophore} + weight \text{ KCIPB}) &= \text{final membrane weight} \\
PL + PU + 10.0 + [(10.0)(496)/(685)(2)] &= 10 / 0.02 \quad \text{(where 685 and 496 represent the molecular weights of ionophone and KCIPB, respectively)} \\
(3/2)PU + PU + (10.0 + 3.62) &= 500 \\
(5/2)PU &= 486 \\
PU &= 194 \text{ mg and PL} = 292 \text{ mg}
\end{align*} \]

KCIPB is added from a 10.0-mg/mL solution in THF, i.e., 0.36 mL. The total volume of THF required is \((500 \times 0.00542)\) or 2.71 mL. Therefore, \((2.71 - 0.36)\) or 2.35 mL more THF is added to the membrane mix. A small magnetic stirrer (2 x 10 mm) is added to the vial and the mixture is capped and stirred for 90 to 120 minutes or until a clear, homogeneous solution is obtained.

A2.6.3 Membrane Fabrication

Membranes were fabricated under a fume hood on a turning spindle by applying the membrane starting solution to the spindle with a small brush. The spindle’s ends were suspended between two bearings and turned at 250 ± 50 rpm by means of a small electric motor with speed controlled by a dual regulated power supply (Model LPD 421FM, Lambda Electronics, Melville, NY). The spindle itself was a 1.3 mm diameter x 200 mm long stainless steel rod (O-SWGX-550) covered with thin-wall, 15-gauge Teflon tubing (ASTT 15-10), both from Small Parts Inc., Miami Lakes, FL. Two 5-mm sections of unplasticized PVC tubing, inside diameter 1/16 inch and wall 1/32 inch (T6034-1 from Scientific Products, Bedford, MA) were fitted as stops to restrict the flow of liquid membrane material. The finished membrane inside diameter was about 1.5 mm and length was from 16 to 20 mm.

1. The liquid membrane material was taken up on a small brush and applied in a smooth, sweeping motion at 10- to 15-minute intervals to allow for drying as THF evaporated between each application. While not in use, the brush was held in a small test tube of THF in such a way that the bristles were completely submerged. Multiple applications (10 to 15) were required to build up the membrane wall; its thickness varied with the amount of material applied and ranged between 0.3 to 0.5 mm.

2. The membrane was turned for one hour after the final application and then allowed to stand overnight in the hood without turning.

3. On the following day, the membrane and attached PVC ends were removed from the spindle and the ends cut off.

4. The membrane was stored on a steel rod at room temperature in a clean environment until used.

5. Membranes were mounted for use between the two tapered glass nipples separated by a 10-mm gap in the ISE half-cell as shown in Figure A1.

6. The membrane is held firmly to the glass nipples and nothing further is needed to maintain this attachment over long and continuous exposure to the filling solution.
(7) Once installed, the membrane was bathed inside and out only by filling solution containing no Triton X-100® except when actually making measurements with calibrators and serum samples.

A2.6.4 Membrane Testing

After at least 24 hours of hydration in the FS and no Triton X-100® exposure, membranes were tested for response slope toward calcium with the 1.25- and 2.50-mmol/L standards. One expects a 9.257-millivolt difference at 37.00 °C between these two standards if the membrane slope is 100% of the theoretical value. The IFCC document¹⁵ suggests that slopes of 95 to 102% are acceptable, but most membranes made with polyurethane in place of polyvinyl chloride initially test nearer to 97 ± 1% of theoretical between 24 to 72 hours following initial hydration. The longevity of a membrane with slope at or above 95% may extend for many days, but we caution that repeated exposure to serum during the later days of this time period, while not causing the slope to drop below 95% as determined with the aqueous standards, gives a disproportionately lower value for the serum-ionized calcium value. It is therefore recommended that important value assignment measurements on serum samples be made with at least two “young” membranes during their early days of use, when total exposure to serum does not exceed seven days.

A3 Procedures

A3.1 Carryover and Hysteresis Effects

The procedures below have been designed to minimize carryover within the fluid path of the system and the hysteresis effect of the ISE membrane. The introduction of numerous air segments between each and every new sample, standard or serum, reduces carryover within the fluid path of the system. Likewise, repeated introduction of the same standard during slope determinations and the use of a close-bracketing standard before and after each serum sample helps to reduce the hysteresis effect of the ISE membrane. This effect is particularly noticeable when the lower 1.25 mmol/L calibration immediately follows the 2.50 mmol/L calibrator.

In addition, a modification to the protocol for measurements on serum samples (Section A3.3) has been necessary to reduce the effect of protein adsorption on the calcium-selective membrane on ionized calcium recovery in serum. This modified procedure includes the addition of two extra wash steps using the same calibrating solution used to bracket the sample, following each serum exposure. A third introduction of the standard is then used as the bracketing standard.

Table A2 shows the effect of added wash steps on ionized calcium recovery in serum.
Table A2. Results Before and After Wash Steps Added to the Protocol

Results Before Wash Steps Added to the Protocol

Mean of First Day and All Days for Membrane Nos. 144 to 163 (Serum Pool 97A, all values in mmol/L iCa$^{2+}$).

<table>
<thead>
<tr>
<th>Mem. #</th>
<th>Start Day</th>
<th># Days Exposure</th>
<th>Slope %</th>
<th>Designated Comparison Method</th>
<th>1st Day</th>
<th>All Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>144</td>
<td>3/3/97</td>
<td>6</td>
<td>97.0</td>
<td>Mean WRSD N</td>
<td>1.268</td>
<td>0.002</td>
</tr>
<tr>
<td>145</td>
<td>4/4</td>
<td>4</td>
<td>97.5</td>
<td>Mean WRSD N</td>
<td>1.261</td>
<td>0.004</td>
</tr>
<tr>
<td>146</td>
<td>4/17</td>
<td>4</td>
<td>96.0</td>
<td>Mean WRSD N</td>
<td>1.284</td>
<td>0.004</td>
</tr>
<tr>
<td>147</td>
<td>5/2</td>
<td>1</td>
<td>96.0</td>
<td>Mean WRSD N</td>
<td>1.272</td>
<td>0.004</td>
</tr>
<tr>
<td>148</td>
<td>5/7</td>
<td>6</td>
<td>96.0</td>
<td>Mean WRSD N</td>
<td>1.276</td>
<td>0.005</td>
</tr>
<tr>
<td>149</td>
<td>5/16</td>
<td>3</td>
<td>95.5</td>
<td>Mean WRSD N</td>
<td>1.267</td>
<td>0.006</td>
</tr>
<tr>
<td>150</td>
<td>6/4</td>
<td>3</td>
<td>95.5</td>
<td>Mean WRSD N</td>
<td>1.269</td>
<td>0.005</td>
</tr>
<tr>
<td>151</td>
<td>6/20</td>
<td>11</td>
<td>96.0</td>
<td>Mean WRSD N</td>
<td>1.292</td>
<td>0.008</td>
</tr>
<tr>
<td>152</td>
<td>8/21</td>
<td>7</td>
<td>97.8</td>
<td>Mean WRSD N</td>
<td>1.276</td>
<td>0.003</td>
</tr>
<tr>
<td>153</td>
<td>7/16</td>
<td>3</td>
<td>96.4</td>
<td>Mean WRSD N</td>
<td>1.266</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total Samples</td>
<td>52</td>
<td></td>
</tr>
</tbody>
</table>

Day to Day Mean ± SD 1.273 0.009 1.258

Results After Wash Steps Added to the Protocol

<table>
<thead>
<tr>
<th>Mem. #</th>
<th>Start Day</th>
<th># Days Exposure</th>
<th>Slope %</th>
<th>Designated Comparison Method</th>
<th>1st Day</th>
<th>All Days</th>
</tr>
</thead>
<tbody>
<tr>
<td>154</td>
<td>9/11</td>
<td>4</td>
<td>97.2</td>
<td>Mean WRSD N</td>
<td>1.297</td>
<td>0.003</td>
</tr>
<tr>
<td>156</td>
<td>10/22</td>
<td>6</td>
<td>98.2</td>
<td>Mean WRSD N</td>
<td>1.296</td>
<td>0.004</td>
</tr>
<tr>
<td>158</td>
<td>12/2</td>
<td>3</td>
<td>97.5</td>
<td>Mean WRSD N</td>
<td>1.307</td>
<td>0.002</td>
</tr>
<tr>
<td>161</td>
<td>12/9</td>
<td>4</td>
<td>97.0</td>
<td>Mean WRSD N</td>
<td>1.291</td>
<td>0.002</td>
</tr>
<tr>
<td>162</td>
<td>12/15</td>
<td>1</td>
<td>97.0</td>
<td>Mean WRSD N</td>
<td>1.306</td>
<td>0.005</td>
</tr>
<tr>
<td>163</td>
<td>1/6/98</td>
<td>7</td>
<td>98.0</td>
<td>Mean WRSD N</td>
<td>1.299</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Total Samples</td>
<td>31</td>
<td></td>
</tr>
</tbody>
</table>

Day to Day Mean ± SD 1.299 0.006 1.292

The data show a relationship between washing of the membrane following exposure to serum and the recovery of iCa$^{2+}$ in serum. In the absence of the extra wash steps, there is a lowering of the serum iCa$^{2+}$ recovery, relative to when the extra wash steps are included (comparing first-day data). When the extra wash steps are omitted, there is a further lowering of the serum iCa$^{2+}$ recovery when the data for all days are compared to the first-day data. The extra wash steps following serum exposure are necessary to maintain membrane stability and have been included in the protocol of Section A3.3. At present, serum pool 97A is used to monitor every membrane with time to detect the first sign of the small, but progressive drop in membrane activity as it reacts to repeated exposure to sera. Seven days of use in serum appears to be a safe recommendation at this time.
A3.2  Response Slope Determination with Working Calibrators

(1) Aqueous calibrator is introduced by removing the bridge line with its bent glass end from the saturated KCl solution of the reference half-cell and fitting the glass end into a small, plastic suction line.

(2) A peristaltic pump is adjusted to pull fluid at about 5 mL/minute through the entire fluid path.

(3) The 1.25 mmol/L aqueous calibrator is first pulled through the 8- to 10-cm stainless steel tube on the distal ISE side of the fluid path.

(4) Initially, ten small air segments are introduced to help flush out the preceding sample by quickly moving the steel tube in and out of the sample to be introduced. The entire fluid path is then filled with the calibrator and the pump stopped.

(5) A small clamp is then applied just above the steel rod and the glass bridge end is detached from the pump line. Any small bubbles in the junction’s tip are flushed out and the junction is reinserted into the saturated KCl of the reference half-cell.

(6) At this time, the end of the glass bridge points upward and the liquid-liquid junction must be at least 1 cm below the surface of the KCl and 1 cm away from the tip of the calomel reference electrode.

(7) The millivolt signal vs. time is recorded for three minutes.

(8) The 1.25-mmol/L calibrator is introduced again with flushing air segments, as above, and the signal is again captured for three minutes. Repeat the 1.25-mmol/L calibrator until it is stable.

(9) The 2.50-mmol/L calibrator is then run and the entire sequence repeated three times.

The mean of the 121- to 180-second readings for the three-minute runs of the 1.25-mmol/L and 2.50-mmol/L calibrators are used to calculate the response slope of the membrane, as given in Section A3.4.1.

A3.3  Measurements on Serum Samples

When a membrane has been shown to have an acceptable response slope (95 to 102% of theoretical) with aqueous calibrators, the next step is to introduce the same sample on which measurements are to be made. Then, a series of alternating calibrator and serum samples should be run, always bracketing the sample with calibrator to minimize the effect of system drift. Following each serum sample, the near-aqueous calibrator should be run three times, the first two runs serving as additional wash steps to reduce the effect of membrane adsorption of protein and other serum components on ionized calcium recovery (Section A3.1) and the third run to serve as the close-bracketing standard.

We recommend the use of ionized calcium standards very near the final concentration of the sample (Section A2.3.5). The 1.25-mmol/L standard should first be used before and after the sample to determine its approximate ionized calcium concentration. Then, a standard that is within ±0.1 to at most ±0.2 mmol/L of the estimate should be used. The procedures for introduction of serum samples and aqueous standards into the measurement system, and for data collection are described in Section A3.2.
A3.4 Calculation of Results

A3.4.1 Slope Calculation

Using the mean of the 121- to 180-second readings for each standard:

\[
\text{Slope} = \frac{\text{mV reading of } 2.50 \text{ mmol/L standard} - \text{mV reading of } 1.25 \text{ mmol/L standard}}{\log_{10}(2.5) - \log_{10}(1.25)}
\]

\[
\% \text{ Slope} = \frac{\text{Slope}}{\text{theoretical slope}} \times 100\% = \frac{\text{Slope}}{30.77} \times 100\%
\]

A3.4.2 Calculation of Serum-Ionized Calcium in mmol/L

Using the mean of the 121- to 180-second readings for serum and the mean of two determinations (preceding and following the serum sample) for the one-point standard, serum-ionized calcium without pH correction is calculated as follows:

\[
[i\text{Ca}^{2+}]_{\text{serum}} = [i\text{Ca}^{2+}]_{\text{standard}} \times 10^{(\text{V reading of serum} - \text{mean of two mV readings of standard}) / \text{slope}}
\]

Ionized calcium concentration in mmol/L with correction to pH 7.40 is calculated as follows:

\[
[i\text{Ca}^{2+}]_{\text{pH corrected}} = [i\text{Ca}^{2+}]_{\text{without pH correction}} \times 10^{(0.24 \times (7.40 - \text{pH})}
\]

Several different functions may be used to correct serum ionized calcium for variations in pH. The reader is referred to the most current edition of NCCLS Document C31—Ionized Calcium Determinations: Precollection Variables, Specimen Choice, Collection, and Handling, and the references contained therein to understand the advantages and disadvantages of each.

A3.4.3 A Note on the Measurement of Ionized Calcium in SRM 956a

Because the ionic strengths of the three levels of SRM 956a are slightly different than 0.160 mol/kg, there is a small error in the assignment of ionized calcium concentrations to SRM 956a using the DCM. Residual differences in the liquid junction potential of the reference half-cell arising from differences in the ionic strengths of the calibrators (0.160 mol/kg) and samples, result in the following errors in the assigned concentrations of ionized calcium given in Table 4.

<table>
<thead>
<tr>
<th>SRM 956a Level</th>
<th>Ionic Strength (mol/kg) (calculated from NIST assigned ion concentrations)</th>
<th>Estimated Error (mmol/L) (of ionized calcium concentrations in Table 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.139</td>
<td>-0.018</td>
</tr>
<tr>
<td>II</td>
<td>0.154</td>
<td>-0.004</td>
</tr>
<tr>
<td>III</td>
<td>0.169</td>
<td>+0.005</td>
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</table>
Appendix B. Certificate of Analysis: Standard Reference Material 956a

National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 956a

Electrolytes in Frozen Human Serum

This Standard Reference Material (SRM) 956a is intended primarily for use in the calibration and standardization of procedures employed in clinical analysis for the determination of specific electrolytes in either diluted or undiluted human serum or plasma. This SRM can be used for standardizing direct-reading ion-selective electrode analyzers [1] and for validating working or secondary reference materials. A unit of SRM 956a consists of six flame-sealed ampules of frozen human serum, two ampules each of three different concentration levels. Each ampule contains \((2.00 \pm 0.04)\) mL of human serum.

Certified Concentrations: The certified concentrations of the serum analytes and density, listed in Table 1, are the mean of results based on measurements using a single primary method. All analytic concentrations are certified as totals. The primary method for potassium and calcium is based on isotope dilution, thermal ionization mass spectrometry [2]. The primary method for the determination of sodium is based on gravimetry after ion exchange separation [3]. The primary method for magnesium and lithium is based on isotope dilution mass spectrometry using inductively coupled plasma mass spectrometry [4]. Density is determined by weighing calibrated pycnometers.

Table 1. Certified Concentrations and Uncertainties

<table>
<thead>
<tr>
<th>Measurand</th>
<th>Level I</th>
<th>Level II</th>
<th>Level III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium (mmol/L)</td>
<td>3.025 ± 0.016</td>
<td>2.570 ± 0.016</td>
<td>2.127 ± 0.009</td>
</tr>
<tr>
<td>Lithium (mmol/L)</td>
<td>2.083 ± 0.005</td>
<td>1.334 ± 0.004</td>
<td>0.570 ± 0.002</td>
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<tr>
<td>Magnesium (mmol/L)</td>
<td>1.441 ± 0.003</td>
<td>0.947 ± 0.002</td>
<td>0.448 ± 0.001</td>
</tr>
<tr>
<td>Potassium (mmol/L)</td>
<td>6.008 ± 0.020</td>
<td>3.985 ± 0.020</td>
<td>2.025 ± 0.008</td>
</tr>
<tr>
<td>Sodium (mmol/L)</td>
<td>121.4 ± 0.3</td>
<td>141.0 ± 0.3</td>
<td>160.9 ± 0.4</td>
</tr>
<tr>
<td>Density (g/mL) at 22 °C</td>
<td>1.0242 ± 0.0004</td>
<td>1.0245 ± 0.0005</td>
<td>1.0251 ± 0.0004</td>
</tr>
</tbody>
</table>

The uncertainty in the certified value is calculated as \(U = k\,u_c\), where \(u_c\) is the combined standard uncertainty calculated according to the ISO Guide [5] and \(k\) is the coverage factor. The value of \(u_c\) is intended to represent, at the level of one standard deviation, the combined effect of uncertainty components associated with the measurement uncertainty and additional Type B uncertainties, such as spike calibration. The expanded uncertainty, \(U = k\,u_c\), is defined as an interval estimated to have a level of confidence of 95%. For users to propagate the uncertainty of calibration when SRM 956a is used as a calibrator, the combined standard uncertainty, \(u_c\), and its associated effective degrees of freedom, \(v_{eff}\), for each level of each analytic concentration are listed in Table 2.

The overall direction and coordination of the technical measurements leading to certification of this SRM were performed by R.L. Watters, Jr. of the NIST Chemical Science and Technology Laboratory.

The support aspects concerning the preparation, certification, and issuance of this SRM were coordinated through the Standard Reference Materials Program by J.C. Colbert.

Gaithersburg, MD 20899
Certificate Issue Date: November 13, 1996

Thomas E. Gills, Chief
Standard Reference Materials Program
Analytical measurements were performed in the NIST Analytical Chemistry Division by S.E. Long, M.S. Rearick, and T.W. Vetter.

Statistical consultation was provided by L.M. Gill of the NIST Statistical Engineering Division.

The development of this SRM is the result of a cooperative research effort between NIST and the NCCLS Subcommittee on Electrolytes. Paul D'Orazio, Chiron Diagnostics, and Gary Graham, Johnson & Johnson Clinical Diagnostics are co-chairmen of the NCCLS Subcommittee on Electrolytes.

Table 2. Combined Standard Uncertainties (mmol/L) and Effective Degrees of Freedom

<table>
<thead>
<tr>
<th>Analytes</th>
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<th>Level III</th>
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<tr>
<td></td>
<td>$u_c$</td>
<td>$v_{eff}$</td>
<td>$u_c$</td>
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<tr>
<td>Calcium</td>
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<td>Lithium</td>
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<td>Magnesium</td>
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<td>Potassium</td>
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<td>12</td>
<td>0.0089</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.15</td>
<td>26</td>
<td>0.16</td>
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</tbody>
</table>

NOTICE AND WARNINGS TO USERS

SRM 956a IS INTENDED FOR IN-VITRO DIAGNOSTIC USE ONLY. THIS IS A HUMAN SOURCE MATERIAL. HANDLE PRODUCT AS A BIOHAZARDOUS MATERIAL CAPABLE OF TRANSMITTING INFECTIOUS DISEASE. The supplier of this serum has reported that each donor unit of serum or plasma used in the preparation of this product has been tested by an FDA approved method and found non-reactive/negative for HIV-1 & 2 antibodies, HbsAg, HCV, and syphilis. However, no known test method can offer complete assurance that hepatitis B virus, hepatitis C virus, HIV, or other infectious agents are absent from this material. Accordingly, this human blood-based product should be handled at the Biosafety Level 2 or higher as recommended for any POTENTIALLY INFECTIOUS HUMAN SERUM OR BLOOD SPECIMEN in the Centers for Disease Control/National Institutes of Health Manual [6].

Stability and Storage: The serum is shipped frozen (on dry ice) and, upon receipt, should be stored frozen until ready for use. A freezer temperature of -20 °C is acceptable for storage up to one week. If a longer storage time is anticipated, the material should be stored at or below -50 °C. The SRM should not be exposed to sunlight or ultraviolet radiation. Storage of thawed material at room or refrigerator temperature may result in changes in the analyte concentrations.

Instructions for Use: Place the ampule to be used inside another container, such as a plastic test tube, to ensure containment of the serum in case the ampule cracks. Each ampule should be inspected carefully for circular cracks at the base. If the ampule is cracked, it should not be used. The serum in intact ampules should be thawed, warmed to room temperature, and mixed by inverting gently at least five times before sampling.

Expiration of Certification: The certification of this SRM is valid until the date printed on the exterior package label, within the measurement uncertainties specified, provided the SRM is handled and stored in accordance with the instructions given on the certificate (see Storage and Instructions for Use). If there is a significant change in any of the certified values purchasers will be notified. Return of the attached registration card will facilitate notification.
SOURCE AND PREPARATION OF SERUM POOLS

SRM 956a was prepared by the Diagnostics Group, Bayer Corporation, Middletown, VA. The material SRM 956a was prepared from normal human serum and its appearance is a clear amber liquid free of particulate matter. Donor units were collected, and allowed to clot for a minimum of two hours at room temperature using no additives to assist in the clot process. The serum pool was frozen at -20 °C, thawed, and filtered through an Avicel Cellulose slurry under vacuum to remove fibrin. Gentamicin sulfate was added as an antibacterial agent. The filtered base pool was diluted with a sodium bicarbonate solution to adjust the potassium level. The plasma was then filtered through a pre-sterilized 0.22 μm filter. The appropriate amounts of chloride salts were added to the Level I and Level III subpools to adjust the concentrations of sodium, potassium, calcium, magnesium, and lithium to the desired levels. The Level II subpool was made from equal amounts of the Level I and Level III subpools. The pH was adjusted to 7.5 at 25 °C. Finally, 2.0 mL aliquots of each subpool were dispensed into 6.0 mL cryogenic glass ampules flushed with an inert gas plus 5 % CO₂ overlay, flame sealed and stored at -50 °C.

REFERENCES

Appendix C. Interlaboratory Study of Ionized Calcium in NIST SRM 956a—Protocol

All serum pools used in this interlaboratory study have been tested and found to be free of both HIV and hepatitis B surface antigen. However, these materials should still be considered as potentially biohazardous, and universal precautions for handling and disposal should be followed.

C1 Receipt of Samples

Six different serum materials, labeled A through F, will be sent to the laboratory. These represent three levels of SRM 956a and three other serum pools, to be used as “test” pools. Five ampules of each material will be sent, for a total of 30 ampules. Upon receipt, samples should be stored frozen until ready to be used. A freezer temperature of -20 °C is acceptable for storage up to one week. If a longer storage time is anticipated, the material should be stored at -50 °C or below.

C2 Preparation of Instrument

Qualify one ionized calcium analyzer for the test. During the test, the analyzer should be used in a manner which is considered representative of how the system is used in the field. This includes frequency of calibration and quality control. Try not to make the system “perfect,” but representative.

C3 Preparation of Samples (Preanalytical)

Because of the influence of pH on ionized calcium, it is important that the samples are thawed and re-equilibrated with the gas in the ampule headspace using the carefully controlled conditions given below.

(1) Remove samples from freezer and thaw at ambient temperature for 1 hour and 40 minutes. (NOTE: Ambient temperature must be between 20 to 24 °C.)

(2) During the first few minutes of thawing, inspect ampules carefully for cracks or breaks. Ampules which are cracked or broken should be discarded and omitted from the data set.

(3) After the 1 hour and 40 minute thawing period, shake each ampule vigorously for 10 seconds with an up and down motion along the cylindrical axis to create foam.

(4) Wait an additional 30 minutes after shaking, then begin analyzing the samples.

C4 Analysis of Samples

The sample analysis procedure is described as follows:

(1) Record the date and starting time for analysis on the data sheet.

(2) Analyze the ampules in the random order shown on the data sheet, starting down the left column. For each ampule, record the specific number of the ampule used, example: B - 75.

(3) Open the ampule and aspirate the sample from as close as possible to the bottom of the ampule. The sample must be introduced into the analyzer within one minute of opening the ampule. Make only one measurement per ampule. In the event of an instrument malfunction or other type of interruption, the same sample may be analyzed again, provided the ampule has not been opened longer than one minute.
(4) Record the ionized calcium and pH (if available) on the data sheet.

(5) If it is not possible to aspirate the sample directly from the ampule into the analyzer for the particular system in use, the sample may be aspirated into a syringe, while minimizing contact with air. Again, the sample should be analyzed within one minute of opening the ampule.

(6) Record the finish time of the analysis.
Appendix D. List of Interlaboratory Study Participants (May 1996)

<table>
<thead>
<tr>
<th>Organization</th>
<th>Contact</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVL Scientific Corp.</td>
<td>Randy Byrd/Gerri Priest</td>
</tr>
<tr>
<td>Baxter Lytening Systems</td>
<td>Dianne Pistone</td>
</tr>
<tr>
<td>CHU Necker-Enfants Malades, France</td>
<td>Charles Sachs</td>
</tr>
<tr>
<td>Ciba Corning Diag. Corp.</td>
<td>Paul D’Orazio</td>
</tr>
<tr>
<td>Danbury Hospital</td>
<td>Sal Sena</td>
</tr>
<tr>
<td>Euro-Trol B.V., The Netherlands</td>
<td>Anton Maas</td>
</tr>
<tr>
<td>Fresenius AG, Germany</td>
<td>Wolfgang Schmehl</td>
</tr>
<tr>
<td>i-Stat Corp.</td>
<td>Nina Peled</td>
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<td>Instrumentation Laboratory</td>
<td>Angelo Manzoni</td>
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<tr>
<td>Mallinckrodt Sensor Systems</td>
<td>Sohrab Mansouri</td>
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<td>Mayo Clinic</td>
<td>Mary Burritt</td>
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<td>Medica Corp.</td>
<td>Photius Makris</td>
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<td>National Institutes of Health</td>
<td>Nadja Rehak</td>
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<td>NOVA Biomedical</td>
<td>Wayne Lambert</td>
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<tr>
<td>Ospendale Niguarda Ca’Granda, Italy</td>
<td>Francesco Zoppi</td>
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<tr>
<td>Radiometer America</td>
<td>Paul Black/Jesper Wandrup</td>
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<tr>
<td>Univ. Of Linkoping, Sweden</td>
<td>Lasse Larsson</td>
</tr>
<tr>
<td>Univ. Texas Med. Branch</td>
<td>Tony Okorodudu</td>
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### Appendix E. Uncorrected Data from Interlaboratory Study (Section 8.3) (all data in mmol/L)

<table>
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<tr>
<th>Lab</th>
<th>Mean</th>
<th>SD</th>
<th>Mean</th>
<th>SD</th>
<th>Mean</th>
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<td>0.004</td>
<td>1.12</td>
<td>0.005</td>
<td>0.97</td>
<td>0.005</td>
<td>1.17</td>
<td>0.000</td>
<td>1.18</td>
<td>0.008</td>
</tr>
<tr>
<td>16</td>
<td>1.69</td>
<td>0.009</td>
<td>1.36</td>
<td>0.007</td>
<td>1.08</td>
<td>0.012</td>
<td>0.96</td>
<td>0.004</td>
<td>1.18</td>
<td>0.004</td>
<td>1.20</td>
<td>0.009</td>
</tr>
</tbody>
</table>

**Code to the Materials:**
- A - SRM 956a Level I
- B - SRM 956a Level II
- C - SRM 956a Level III
- D,E,F - ionized calcium test pools
Appendix F. SRM 956a Process Flow Chart

THAW AND POOL SERUM

SLOWLY ADJUST pH TO 7.4 WITH GLACIAL ACETIC ACID

ADD 3.0 g/L AVICELL CELLULOSE

MIX 30 MINUTES

FILTER TO REMOVE FIBRIN STRANDS AND CELLULOSE

ADD 50 mg/L GENTAMICIN

FILTER THROUGH 0.22 MICRON FILTER

DRAW SAMPLE FOR ANALYSIS

DILUTE 1:1 (GRAVIMETRICALLY) WITH 24 mmol/L NaHCO₃ (K⁺ CONC = 2.0 ± 0.1 mmol/L)

ULTRA FILTER USING OSMONICS 20,000 min MEMBRANE CARTRIDGE TO T PROTEIN = 70 ± 5 g/L.
ASSAY FOR Na,K,TCa,TMg & Li

GRAVIMETRICALLY SPLIT INTO 2 EQUAL POOLS

SPIKE TO THE FOLLOWING TARGETS USING ACS GRADE CHLORIDE SALTS
Na= 120 ± 2mmol/L K = 6.0 ± 0.2mmol/L
TCa= 3.0 ± 0.2mmol/L TMg= 1.5 ± 0.1mmol/L
Li= 2.0 ± 0.1mmol/L

GRAVIMETRICALLY MIX EQUAL AMOUNTS OF EACH POOL TO FORM A MID POOL

GRAVIMETRICALLY MIX EQUAL AMOUNTS OF EACH POOL TO FORM A MID POOL

FOR EACH OF THE THREE POOLS

0.22 MICRON FILTER

0.22 MICRON FILTER

0.22 MICRON FILTER

EQUILIBRATE WITH AND FILL UNDER 5% CO₂/95% NITROGEN GAS

FILL 2.0 mL INTO 5 mL PRESCORED 33 EXPANSION FLINT AMPULES

BULK PACKAGE & FREEZE AT -50°C OR BELOW

SPIKE TO THE FOLLOWING TARGETS USING ACS GRADE CHLORIDE SALTS
Na= 160 ± 2mmol/L K = 2.0 ± 0.2mmol/L
TCa= 3.0 ± 0.2mmol/L TMg= 1.5 ± 0.1mmol/L
Li= 2.0 ± 0.1mmol/L

SPIKE TO THE FOLLOWING TARGETS USING ACS GRADE CHLORIDE SALTS
Na= 120 ± 2mmol/L K = 6.0 ± 0.2mmol/L
TCa= 3.0 ± 0.2mmol/L TMg= 1.5 ± 0.1mmol/L
Li= 2.0 ± 0.1mmol/L
Summary of Comments and Subcommittee Responses

C39-P: A Designated Comparison Method for the Measurement of Ionized Calcium in Serum; Proposed Standard

General

1. The standard makes little, if any, mention of the effects of protein or lipid in a serum sample on the water content of the sample. Given the ISE will measure concentration in the plasma water, this concentration bears a variable relation to the concentration in the whole sample, when expressed as mmol/L, depending on the amounts of lipid or protein present. Since aqueous standards are used to calibrate the method, no account is made for this effect in the serum sample. Although this problem cannot be overcome in practice, it should be noted somewhere in the standard with respect to actual serum sample measurement, that the concentrations measured are plasma water concentrations.

- The subcommittee agrees that the commenter makes a valid point. A paragraph regarding this issue has been added to the end of Section 4.

Section 5

2. The definitions of “Ionized calcium” (page 3) and “Total calcium” (page 4) are not analogous. The former is descriptive, the latter refers to concentration. “Total calcium” would be better defined as “The totality of calcium species in plasma including…” Alternatively, the definitions could be modified to “Total calcium concentration” and “Ionized calcium concentration.” For these definitions, the current “Total calcium” text would remain as the “Total calcium concentration” text, but the “Ionized calcium concentration” text would become “The concentration of calcium ions in an aqueous solution of 0.160 mol/kg whose calcium activity is the same as that of plasma. The plasma activity is contributed to by calcium that is not bound by protein or other species.” This has a real advantage in that it defines clearly the way in which the assay is calibrated. This is an extremely common area of ignorance of analysts.

- The definition for “ionized calcium” has been revised by replacing “portion” with “concentration.”

Appendix A1.3.1

3. I felt it would be helpful to give examples of some of the electrometers which might be used instead of just providing references and specifications. Unless I missed it, there was no mention of the particular electrometer used in the NCCLS work. Perhaps the authors wish to avoid endorsing any commercial equipment, but I noticed many other citations of suppliers of materials.

- A1.3.1 has been revised with the following reference: World Precision Instruments, Sarasota, FL (Model VF4, four-channel buffer amplifier).

Appendix A2.5

4. The document is well written, thorough and appears to provide all the information needed to follow the proposed procedures. I should have preferred to see the structure for the ionophore ET1001 shown in the text. As it is, I found it by referring to a catalog.

- The subcommittee appreciates the comment. The following source has been added after the second sentence of Note “a” in Section A2.6.1: “ETH 1001 has recently become available from another commercial source, Selective Technologies Inc., Flint, MI and is sold under the trade name CA 1001.” The chemical structure “ETH 1001” has also been inserted into this section.
Summary of Delegate Comments and Responses

C39-A: A Designated Comparison Method for the Measurement of Ionized Calcium in Serum; Approved Guideline

General

1. Under the Introduction, I would like to have seen a brief discussion of Clinical Usefulness.

   • References 1-11, cited in the Introduction, cover the clinical usefulness of ionized calcium in detail. The document is already lengthy and to repeat this information in the text would only make it longer.

2. The serum matrix effect on the Ion selective Calcium Membrane seems to give falsely high readings of ionized calcium. This bias seems to be confirmed from the data given in Table A2, where the serum ionized calcium reading is higher after an extra wash step compared to the measurement with a normal wash.

   The extra wash step may remove the protein coating from the electrode membrane surface and the reading is then lowered on the calibrant compared to a reading on the calibrant immediately after exposure to serum.

   It is my experience that the protein build-up on the Ionselective is instantaneous and it can be removed by washing the membrane for some time. The effect is depending on the preparation of the membrane, the storage condition of the membrane between measurement and the age of the membrane in wet or dry stage.

   In commercial instruments the serum effect is eliminated in two ways: 1) The electrode is calibrated immediately after measurement on serum, 2) The ionselective is covered by a hydrophilic membrane.

   • The serum matrix effect you describe is well known to those with experience in working with ion selective electrodes. In many years of studying the DCM presented in C39-A, we know that serum protein adsorption on the membrane surface will lower the recovery of ionized calcium in serum after the membrane has reached a condition of pseudoequilibrium, with respect to protein adsorption, between samples and calibrating standards. This is because the potential measured in the calibrating standard is increased to a more positive value following exposure of the membrane to serum. However, this adsorbed protein layer is in an unstable condition. The calibrating solution immediately run after the serum sample always shows more negative potential drift during the measurement than a calibrating standard which is run after two additional washes. This is indicative of protein desorbing from the surface of the membrane during measurement of the calibrating solution immediately following the sample, leading to a false calibration. In developing this DCM, we have chosen to eliminate the protein adsorption effect on calibration of the system by adding extra wash steps to the sample measurement protocol. Therefore, the standards which are used to bracket the sample and calibrate the system are performed in a method which is as accurate as possible, without the added variable of protein adsorption. The ionized calcium data for NIST SRM 956a using the DCM were obtained in this manner.

3. The red cell effect is not addressed in this standard. Most commercial instruments measure ionized calcium on whole blood. In normal blood samples, the red cell effect at the liquid junction will cause a positive bias of 1 mV corresponding to a reading 8 percent too high. The red cell effect is dependant on the hematocrit of the blood; increasing with increasing hematocrit.
The Designated Comparison Method described in Appendix A of this document is used to measure the concentration of ionized calcium in serum, not whole blood. However, the C39-A method can be used to reduce interlaboratory variations for the measurement of ionized calcium in whole blood as well as serum. The DCM was used to assign ionized calcium concentrations to a commercially available reference serum (SRM 956a). This reference serum may be used to standardize ionized calcium analyzers in the field, thus reducing the interlaboratory bias for ionized calcium measurements in serum as well as whole blood. Whole blood may introduce an additional bias caused by the presence of red cells; however, the red cell effect on the liquid-junction potential is only one variable of many leading to bias from one commercial system to another. Standardization of ionized calcium to SRM 965a, with concentrations assigned by DCM, will eliminate the vast majority of these differences.

4. Residual liquid junction potentials: The estimated errors for the ionized calcium values in SRM 956a given in the table in Section A3.4.3 seem to be wrong because the residual liquid junction potential between serum with a high concentration of immobile protein anions and the calibrant with the highly mobile chloride-ion is much higher than indicated in the table.

- The differences mentioned with respect to the mobility of protein anions versus chloride are minimized by the use of an open, saturated KCl salt bridge. Our calculations have shown that the errors presented in Section A3.4.3 are primarily due to differences in ionic strength among the levels of SRM 956a (0.139 mol/kg in Level I to 0.169 mol/kg in Level III) and the calibrating solutions which are at 0.160 mol/kg).
Related NCCLS Publications*

C29-A  
**Standardization of Sodium and Potassium Ion-Selective Electrode Systems to the Flame Photometric Reference Method; Approved Standard (1995).** This document provides recommendations on the expression of results of ion-selective electrode measurement of sodium and potassium ion activities in undiluted serum, plasma, or whole blood in clinical practice.

C31-A  
**Ionized Calcium Determinations: Precollection Variables, Specimen Choice, Collection, and Handling; Approved Guideline (1995).** This document addresses preanalytical considerations — such as patient condition, specimen choice, collection, and handling — that can influence accuracy and clinical utility of ionized calcium measurements.

M29-A  
**Protection of Laboratory Workers from Instrument Biohazards and Infectious Disease Transmitted by Blood, Body Fluids, and Tissue; Approved Guideline (1997).** This document provides guidance on the risk of transmission of hepatitis viruses and human immunodeficiency viruses in any laboratory setting; specific precautions for preventing the laboratory transmission of blood-borne infection from laboratory instruments and materials; and recommendations for the management of blood-borne exposure.

NRSCL8-A  
**Terminology and Definitions for Use in NCCLS Documents; Approved Standard (1998).** This document provides standard definitions for use in NCCLS standards and guidelines, and for submitting candidate reference methods and materials to the National Reference System for the Clinical Laboratory (NRSCL).

NRSCL13-P  
**The Reference System for the Clinical Laboratory: Criteria for Development and Credentialing of Methods and Materials for Harmonization of Results; Proposed Guideline (1995).** This document provides procedures for developing and evaluating definitive methods, reference methods, and reference materials to provide a harmonized clinical measurement system.

* Proposed- and tentative-level documents are being advanced through the NCCLS consensus process; therefore, readers should refer to the most recent editions.